A review on potential enzymatic reaction for biofuel production from algae

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ABSTRACT

Increasing number of population, advanced technology and economics growth somehow has caused energy depletion. The development of biofuel such as biodiesel, bioethanol and biogas is extremely needed to overcome these crises. Biofuel is derived from biological sources or biomass which are more environmental friendly, less toxic, reduce greenhouse gas emission and less cost. Algae comes from the third generation product of both biodiesel and bioethanol. Algae based biofuel has various advantages such as non-toxic, does not require fresh water to grow, higher growth rate, biodegradable and not used arable land. Thus, this review summarizes enzymatic reaction for production of all biofuels; biodiesel and bioethanol. The enzymatic reaction is safe, less contaminating and seems to produce higher yield of biofuel compared to chemical reaction. Overall finding of this study suggests that immobilization method and efficiency of the enzyme is the main factor in biofuel production. Finally, further studies are recommended to overcome the major constraint of high enzyme cost by improving the immobilization technique and processes.

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Contents

1. Introduction

It is not a secret that fossil fuels are the main source of energy for the world and attract millions of users in many applications and sectors such as business, transportation, research, and industries. There are three major forms of fossil fuels: coal, oil and natural gas which were formed hundred millions years ago. However, fossil fuel is not infinite and there are many challenges faced by societies such as energy security, increase of oil price, resource depletion, and climate change that lead researchers to search for new and attractive sources to replace the fossil fuels [1]. Biofuel is one of the alternatives derived from various renewable biological sources such as algae, soybean, jatropha, corn, palm, coconut, rice bran, linseed, jojoba, castor and waste [2]. Production and usage of biofuel are not new. Vegetable oil has been used in an engine during 1930s in emergency cases [3]. Currently, three types of biofuels have been discussed extensively by other researchers; which are biodiesel, bioethanol and biohydrogen. There are many improvements and research focused to enhance the quality of a biofuel that is feasible and safe for consumers. According to the

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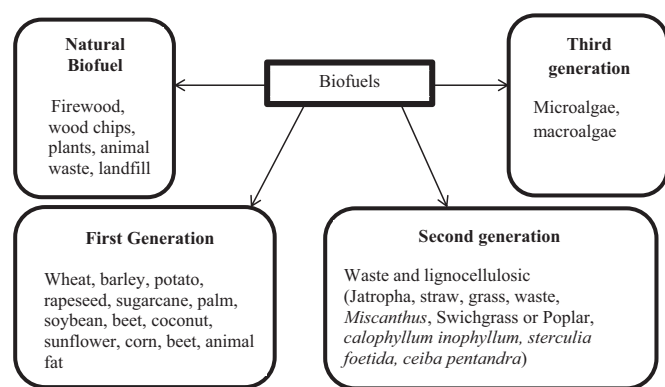 *E-mail addresses:* onghc@um.edu.my, ong1983@yahoo.com (H.C. Ong).

Fig. 1. Generation of biofuels.

Table 1
Comparison of elemental and chemical content of biodiesel and diesel [19].

Components	Biodiesel content (%)	Diesel content (%)
Carbon	79.6	86.4
Hydrogen	10.5	13.6
Oxygen	8.6	–
Nitrogen	1.3	–
C/H	7.6	6.5
n-aliphatics	15.2	67.4
Olephenics	84.7	3.4
Aromatics	–	20.1
Naphtens	–	9.1

reports of international agencies roughly 53% increase in the demand of energy is expected by the year 2030 [4]. According to this statistic, world consumption of petroleum is 89.41 million barrels per day in 2012 [5] and will increase to 136.80 million barrels per day by 2030. Malaysia is the second largest oil and natural gas producer in Southeast Asia, also the second largest exporter of liquefied natural gas globally. Malaysia's production of petroleum is expected to reach 700 thousand barrels per day and consumption of petroleum, 598 thousand barrels per day in 2014 [5].

Biofuels has several classifications, namely natural biofuel, first generation, second generation and third generation as shown in Fig. 1. Natural biofuels are generally derived from organic sources such as vegetables, animal waste and landfill gas [6]. The natural biofuels is commonly used for cooking, heating, brick kiln or electricity production. On the other hand, first generations of biofuel are derived from edible feedstock like wheat, palm, corn, soybean, sugarcane, rapeseed, oil crops, sugar beet and maize [7]. It is expected that the growth in production and consumption of biofuels are increasing, but their impacts towards meeting the overall energy demands in the transport sector will remain limited. This is mainly because of the competition with food and fiber production for the use of arable land, regionally constrained market structures, lack of well managed agricultural practices in emerging economies, high water and fertiliser requirements, and a need for conservation of bio-diversity [8]. Thus, the first generation is claimed to be not very successful since it affects food security and global food markets.

Furthermore, waste and dedicated lignocellulosic feedstocks such as *Miscanthus*, jatropha, *Sterculia foetida*, *Ceiba pentandra*, Swichgrass or Poplar are known as second generation of biofuels [7,9]. This generation of biofuels brings many more advantages compared to the first generation due to higher yield and lower land requirement. The major drawback is the lack of efficient technologies for commercial applications [7]. Third generation of biofuels uses macro- and micro-algae as feedstock and it is seen as a technically viable alternative energy approach that may overcome the major drawbacks associated with the first and second generation biofuels [8]. Numerous advantages have been highlighted for the fuel production from algae such as high growth rate, high efficiency CO_2 mitigation, less water demand than land crops and more cost effective farming [10].

To date, most reviews emphasized on the production of biofuel, technologies [8], processes [11] and potential of variable feedstocks such as algae for biofuel production [12]. These reports focus merely on the advantages of the feedstocks, techniques, processes for biodiesel and bioethanol. As yet, no review has been written about enzyme-catalyzed reaction for biodiesel production from microalgae. Therefore, this review is aimed to fill this gap and summarize about the extraction of biofuels such as biodiesel and bioethanol from algae by enzyme-catalyzed reaction.

2. Biodiesel

Biodiesel is usually defined as methyl (or ethyl) esters of fatty acids obtained by transesterification (alcoholysis) of triglycerides. Typically, biodiesel encompasses alkyl fatty acid (chain length C_{14}–C_{22}) esters of short-chain alcohols, primarily, methanol or ethanol. Biodiesel has the most compatible characteristics with the fossil fuel due to its higher heating value, flash point, cetane number and kinematic viscosity [13]. Biodiesel is one of the most demanding fuels to support limited supply of fossil fuels in future. In addition, the utmost characteristics of biodiesel is abundance; it is environmental friendly, its reduces net carbon-dioxide emissions by 78% [14–16], it is renewable, non-toxic, non-flammable, and biodegradable [4,17,18]. Moreover, higher flash point of vegetable oils, makes their storage, transportation and handling easier. Biodiesel is also 66% better than petrodiesel when used as lubricant [13].

Biodiesel has more polar structure based on the oxygen level compared to diesel as shown in Table 1. Therefore biodiesel has higher viscosity for lower heating value compared to diesel fuel [19]. Biodiesel can be used in its pure form or mixed with certain proportions of diesel such as B20 which contains 20% biodiesel and 80% diesel.

There are several methods to extract and esterifies vegetable oil or animal fats into biodiesel. It could be extracted chemically using solvent such as hexane or benzene [14,20]. It can also be extracted physically either by using oil press, osmotic shock or ultrasound [21]. In order to esterify the oil into fatty acid methyl ester (FAME), few methods have been applied and studied to enhance the efficiency of biodiesel. Among the popular method are acid-catalyzed transesterification, alkali-catalyzed transesterification, enzymatic transesterification followed by supercritical ethanol [22], supercritical carbon dioxide, microwave, and ultrasound transesterification [23]. The latest technology in biodiesel production including wet lipid extraction [24], continuous heterogeneous catalyzed process or McGyan® process [20,25] and enzymatic conversion in ionic liquid [26]. In McGyan® process reported by Krohn et al. a biomass of wild culture, Kelp, *Dunaliella tertiolecta* and *Nannochloropsis oculata* was converted to biodiesel at 85% efficiency [20].

The conventional process for biodiesel production is transesterification of oil and alcohol using catalysts or supercritical conditions with or without presence of catalyst. Yet, the usage of homogeneous catalyst or alkaline-based solid catalyst (e.g metal oxides) tend to initiate the soap generation during the reaction when oil sources contain water and free fatty acids and deactivated the catalyst [7]. Consequently, dewatering of oil sources is crucial and required to ensure the efficiency of transesterification process [25]. Moreover, chemical method has high energy

consumptions, difficulty in recovering the glycerol and significant produce amount of alkaline waste water [27]. As the downstream processing problems caused by chemical transesterification, enzyme-catalyzed (lipase) transesterification is preferred method for biodiesel production. Enzyme-catalyzed reactions are consume less energy intensive, does not promote side reactions and more environmentally friendly [12]. However, the major drawbacks in enzymatic reaction are product contamination with residual enzymatic activity, higher cost of catalyst, short operational life caused by the negative effects of excessive methanol and by product glycerol [28].

Few potential solutions have been proposed to overcome these drawbacks such as immobilization of lipase, solvent engineering, acyl acceptors alteration, methanol stepwise addition and substrate oil sources [29]. Lipase inactivation caused by short chain alcohol could be inhibited via acyl acceptors but the low reaction time and high cost are major constraints for industrial application. Meanwhile, t-butanol is a good solvent of the substrate methanol and commonly applied in industry. Solvent engineering could reduce the reaction time, but it must be separated from the products. This extra step significantly increases the energy consumption of the whole process [30]. The methanol stepwise addition is the common choice since it not only avoids the methanol toxic effect, but also expand the contact area of oil and methanol to achieve a high yield using simple operating conditions [28,31].

2.1. Use of lipase in biodiesel production

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important biocatalysts because of their excellent biochemical and physiological properties [32]. Lipases can be found in nature and produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, are the most widely used in biotechnological applications and organic chemistry. Biodiesel production is one of the impressive applications of lipase. Lipase catalyzed biodiesel production was firstly reported by Mittelbach [19]. *Burkholderia cepacia* lipase (BCL) is the most widely reported in biodiesel production in isooctane and solvent-free system [29]. Other bacterial lipases reported are *Pseudomonas fluorescens* lipase, *Pseudomonas cepacia* lipase and *Chromobacterium viscosum* lipase [27,33]. Moreover, Novozyme 435 from *Candida antarctica* lipase is the most widely used yeast lipase. Another lipase from fungi is *Thermomyces lanuginous* lipase (TLL), *Rhizopus oryzae*

lipase (ROL), *Penicillium expansum* lipase (PEL) and *Geotrichum sp.* lipase (GSL) [33].

Lipases are hydrolases, which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol [32]. Lipases have very low solubility, with substrates of long chain triacylglycerols. Under micro-aqueous conditions, lipases possess the unique ability to carry out the reverse reaction, leading to esterification, alcoholysis and acidolysis [32]. Another advantages of lipase in biodiesel production are high efficiency; complete conversion of free fatty acids (FFA) to methyl/ethyl esters, specific reaction, less energy consumption, mild reaction conditions, low temperature, and reduces the formation of side products and wastes [34].

Transesterification process is carried out in the presence of catalyst, either chemical or enzymatic catalyst to accelerate the reaction. Enzymatic catalyst is always superior in transesterification reaction due to mild reaction condition, reusable of the catalyst, easy separation of products and produces high quality product [35,36]. Lipase is important enzyme catalyst that catalyzes esterification and transesterification reaction to yield methyl esters. In transesterification, triglycerides will be catalyzed into FAME and glycerol as the by product with a presence of lipase as shown in Fig. 2.

There are several factors affecting enzymatic transesterification process namely pH, concentration of substrates, temperature, activity of the enzyme and spacing between the enzyme molecules and the substrate. Each enzyme works best at their optimum condition and varies depending on its origin. The specialities of enzyme are non-toxic, reusable, and sometimes, it can be reused till 10th cycle. However, if the enzyme mixes with the product or the solvent, it requires more downstream processing to separate them. Moreover, free alcohols such as excess methanol and glycerol produced, will stimulate dehydrogenases during the process and consequently inhibit the catalytic activity [35].

Commonly, lipases will be immobilized in four methods to maintain lipase stability and avoid the weaknesses of enzymatic reaction, which included adsorption, covalent binding, entrapment, encapsulation and cross-linking [27] as shown in Fig. 3. The advantages of immobilized lipase are presented in Table 2. Adsorption is the attachment of lipase on the surface of the carrier by weak force, such as van der Waals, hydrophobic interactions or dispersion forces [27]. Adsorption is the most widely used method in immobilization because the process is easy and can be operated at low cost. It works under mild conditions without major activity loss. There are two common lipases especially used in large scale industry which uses adsorption method to immobilize. One is the *C. artartica* immobilized on acrylic resin, which is known by its commercial name Novozyme 435. The other is the *Candida sp.* 99–125 lipase immobilized on cheap textile membrane. Biodiesel yield can reach between 90% and 97% by adsorption method in cotton seed [37] and microalgae [26] respectively.

Another approach to immobilize lipase is covalent bonding, whereby the covalent bonds are formed between the aldehyde groups of surface and active amino acid residues on the surface of the enzyme [19]. The strong interactions between lipase and the
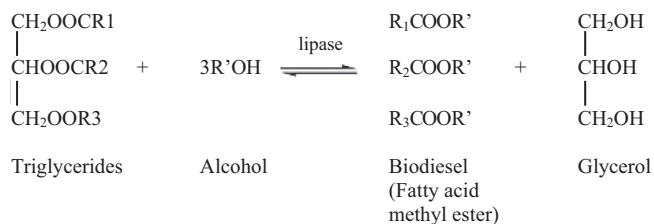
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\begin{array}{ccccccc}
CH_2OOCR1 & & & R_1COOR' & & CH_2OH \\
| & & & & & | \\
CHOOCR2 & + & 3R'OH & \xrightleftharpoons[]{lipase} & R_2COOR' & + & CHOH \\
| & & & & & | \\
CH_2OOR3 & & & R_3COOR' & & CH_2OH \\
\\
\text{Triglycerides} & & \text{Alcohol} & & \begin{array}{c}\text{Biodiesel}\\\text{(Fatty acid}\\\text{methyl ester)}\end{array} & & \text{Glycerol}
\end{array}
$$

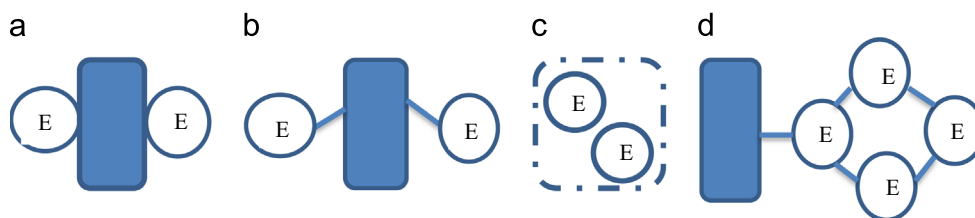**Fig. 2.** Enzymatic transesterification reaction.

Fig. 3. Schematic diagram of enzyme immobilization method: (a) adsorption; (b) covalent binding; (c) entrapment; (d) cross-linking; E=enzyme.

support make enzyme leaching uncommon during the catalytic process. A variety of supports have been used such inorganic materials, natural polymers (agarose, chitin and chitosan), synthetic polymers (hydrophobic polypeptides, nylon fibers) and Eupergit® (made by copolymerization of N,N′-methylenebis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide) for immobilization of lipases by covalent bonding [38,39]. Previously, lipase from *Burkholderia sp. C20* and *Thermomyces lanuginosus* (Lipozym TL) has been applied to microalgae and canola oil, respectively. The biodiesel was obtained at maximum of 97.3% from microalgae. Higher percentage yield of biodiesel is obtained from the stability of lipase enzyme during the reaction. Chitosan, poly [b-(1–4)-linked-2-amino-2-deoxy-D-glucose] is a promising magnetic carrier which is non-toxic, hydrophilic, biocompatible, biodegradable and anti-bacterial, since it has a variety of functional groups which can be tailored to specific applications [40]. Xie and Wang [40] reported a technique for immobilization of *Candida rugosa* lipase on magnetic chitosan microspheres for transesterification of soybean oil. The immobilized enzyme was determined as an effective biocatalyst for the transesterification reaction due to the fact that it gives a good conversion of soybean oil and the activity is retained during four cycles [19].

Entrapment method is based on capturing the lipase within a polymer network that retains the enzyme but allows the substrate and products to pass through. This method can be simply defined as mixing an enzyme with a polymer solution and then crosslinking the polymer to form a lattice structure that captures the enzyme [19]. Entrapment of a lipase entails capture of lipase within a matrix of polymer. *P. cepacia* was entrapped in hydrophobic sol–gel, and the immobilized lipase could catalyze biodiesel production with soybean oil as feedstock. The final conversion was around 67% [27].

Cross-linking is another method for immobilization which is defined as the interaction of a three dimensional network within enzyme, coupling reagent, and carrier [19]. Application of crosslinking method was reported by You et al. [41] in production of biodiesel from jatropha oil. Lipase from *B. cepacia* was immobilized

on modified attapulgite with the best conditions; 10 g jatropha oil, 2.4 g methanol being added at 3 h intervals, 7 wt% water, 10 wt% immobilized lipase, temperature at 35 °C, and time 24 h. The maximum biodiesel yield reached 94% after 24 h under these reaction conditions. After 10 times of lipase reuse, the recorded biodiesel yield was around 89%, corresponding to 94% of that obtained in the first batch, which suggests its potential for industrial applications [41].

Nanomaterials are another advanced technology in immobilization of enzyme. Nanomaterials such as CaO and MgO nanoparticles have been used as biocatalyst carriers or as heterogeneous catalyst in oil transesterification to biodiesel [42,43]. The application of nanomagnetic materials in enzyme immobilization is efficient and the recovery of lipase could be easily and rapidly (within 1 min) completed by the addition of external magnetic fields. It implies that immobilizing lipase onto nanomagnetic materials could be a strategy of enhancing the reusability of lipase. On top of that, another study reported the activity of the lipase immobilized onto carbon nanotubes (CNTs) in transesterification of ethyl butyrate and found that 97% activity of the lipase was retained [42]. Table 3 summarizes the advantages and disadvantages of methods to immobilize enzyme.

Methanol is a common solvent in esterification process and it acts as an acyl acceptor in enzymatic reaction. It inhibits lipase reaction and it should be used in an amount that is an excess amount of the stoichiometric ratio [44]. However, high methanol concentrations are generally toxic to lipase, thereby decreasing its enzyme activity and stability [41,45]. Thus, the method of methanol addition may affect the biodiesel yield. Stepwise addition is recommended to overcome this problem and avoid excess methanol as suggested by many researchers [28,31,33,44]. In addition, liberated glycerol may also inhibit the reaction by limiting substrate and product diffusion, due to its insolubility in oil or organic solvent. Shimada et al. [31] studied the effect of methanol to lipase activity by introducing stepwise alcoholysis system. From the study, two-step batch methanolysis at 30 °C is most effective for production of biodiesel fuel from waste edible oil. Meanwhile, three-step flow reaction will be available if immobilized carrier is destroyed by mechanical agitation. This system is also applicable to batch reaction with free enzyme besides continuous reaction with immobilized enzyme. The application of lipase in biodiesel production is summarized in Table 4.

2.2. Biodiesel from algae by enzymatic transesterification

Currently, microalgae have become the most attractive feedstock for biodiesel because of its high production yield. The third generation of biofuels are focusing on the use of microalgae for the production of biofuels such as biodiesel, bioethanol and biogas for

Table 2
Comparison of free enzyme and immobilized enzyme [19].

Characteristics	Free enzyme	Immobilized enzyme
Price	High	Low
Efficient	Low	High
Activity	Unstable	Stable
Reusability and recovery	Not possible	Possible
Tolerance to temperature, pH	Low	High
Separate from substrate	Difficult	Easy
Separate from product	Difficult	Easy

Table 3
Comparison of methods to immobilize enzyme [19,27].

Method	Advantages	Disadvantages
Adsorption	Worked in mild condition, easy and low cost. Regenerated carrier for several time usage.	Weak interaction between lipase and the the carrier make the immobilized. lipase sensitive to pH, ionic strength and temperature etc. The adsorption capacity is small and the protein might be stripped off from the carrier.
Covalent bond	Thermal and operational stable enzyme.	The laborious preparation of immobilized enzyme might cause lipase to lose its activity. Some coupling reagents are toxic.
Cross-linking	Lipase is stable due to strong interaction between the lipase and the carrier.	The cross-linking conditions are intense and the mechanical strength of the immobilized lipase is low.
Entrapment	The conditions are moderate and applicable to a wide range of carrier and lipases. Fast, cheap and easy.	The lipase is only effective for low molecular weight substrates because it has the mass transfer restriction during the catalytic process.

Table 4
Application of lipase enzyme in production of biodiesel.

Raw material/ oil	Name of enzyme	Lipase origin	Immobilized method	Carrier	Solvent used	Yield/conversion	References
Sunflower	Novozyme 435	*Mucor miehei, Candida antartica*	Adsorption	Macroporous resin	–	–	[33]
Cotton seed	Novozyme 435	*Candida antarctica*	Adsorption	Macroporous resin	*t*-butanol	97%	[37]
Canola	Lipozyme TL	*Thermomyces lanuginosus*	Covalent binding	Polyurethane foams	Methanol	90%	[28]
Soybean	Lipase	*Candida rugosa*	Covalent binding	Magnetic chitosan	methanol	87%	[40]
Soybean	Lipase	*Pseudomonas cepacia*	Covalent binding	Activated magnetic silica nanocomposite Particles	Methanol	54%	[46]
Jatropha	Lipase	*Burkholderia cepacia*	Cross-linking	Modified attapulgite (ATP)	Methanol	94%	[41]
Simulated waste cooking	Lipase	*Candida antarctica, Pseudomonas cepacia*	Adsorption	Ceramic beads	Methanol, *n*-hexane	40% without *n*-hexane	[47]
Sunflower, soybean, waste cooking	Lipase	*Thermomyces lanuginosus*	Covalent binding	Microporous polymeric biocatalyst (Bead, powder, monolithic)	Methanol	63.8% (sunflower), 55.5% (soybean), 50.9% (waste cooking)	[48]
Sapium sebiferum	Lipase	*Pseudomonas cepacia G63*	–	–	Methanol	96.22%	[49]
Waste grease	Lipase	*Thermomyces lanuginosus Lipase* (*TLL*), *Candida antartica Lipase B*	Covalent binding	Iron oxide (magnetic nanobiocatalyst aggregates (MNA))	Methanol	> 97%	[50]
Palm	Novozyme 435, Lipozyme TL IM	*Candida antarctica B, Thermomyces Lanuginous*	Adsorption, covalent binding	Macroporous acrylic resin, silica gel	Dimethyl carbonate (DMC)	90.5%, 11.6%	[51]
Waste, corn	Lipase	*Penicillium expansum*	Adsorption	Resin D4020	Methanol	92.8%,	[52]
Soybean	Lipase	*Candida rugosa* (Amano AY-30)	Adsorption	Polyvinylidene fluoride (PVDF) membrane	Methanol, *n*-hexane	97.2%	[53]
Soybean	Lipozyme TL	*Thermomyces lanuginosa*	Covalent binding	Magnetic nanoparticles	Methanol	> 90%	[54]

Table 5
Advantages of microalgae based biofuel.

Advantages of biodiesel from microalgae	References
High oil content	[12,29,56,57]
Higher biomass production	[56,58,59]
Higher photosynthetic efficiency	[57,60]
Higher growth rate	[12,57,58]
Reduce gas emission through carbon dioxide fixation	[56,58]
Minimal water use	[25,55,56]
No additional land use	[25,55]
No pesticides	[55]
Not required fresh water	[55,56]
No competition with foods	[56]
Easy to scale up	[29]
Non-toxic, contains no sulfur	[58]
Highly biodegradable	[58]

Table 6
Oil content in microalgae [12,29,57].

No.	Microalgae	Oil content (% dry weight)
1	*Botryococcus braunii*	25–75
2	*Chlorella sp.*	
	C. vulgaris	63.2
	C. prototecoides	55–58
	C. pyrenoidosa	2.2
	C. sorokiana	22
3	*Crypthecodinium cohnii*	20–56
4	*Cylindrotheca sp.*	16–37
5	*Dunaliella primolecta*	23
6	*Isochrysis sp.*	25–33
	Isochrysis galbana	14.5
7	*Monallanthus salina*	> 20
8	*Nannochloropsis sp.*	20–35
9	*Neochloris oleoabundans*	35–54
10	*Nitzschia sp.*	45–47
11	*Phaeodactylum tricornutum*	20–30
12	*Schizochytrium sp.*	50–77
13	*Tetraselmis suieca*	15–23
14	*Nannochloris sp.*	31–68

the utmost characteristics of microalgae [7]. Besides having high productivity of oil, microalgae also requires a lower rate of water renewal than terrestrial crops, can be cultivated in brackish water, does not require pesticides, and arable land as field crops do [55]. Other advantages of microalgae in biodiesel production are summarized in Table 5.

Microalgae could be the potential and most economical source of biodiesel because of its high yielding feedstock. Oil content in microalgae may exceed 80% by weight of dry biomass and oil levels of 20–50% are quite common in any microalgae [12,57]. The oil productivity, which is the mass of oil produced per unit volume of the microalgae broth per day, depends on the algae growth rate and the oil content of the biomass. Microalgae with high oil productivities are desired for producing biodiesel. The most suitable triglyceride feedstock for biodiesel production should have a chain length between C14 and C22 and low saturation level [25]. Microalgae oils differ from most vegetable oils in terms of its abundant polyunsaturated fatty acids with four or more

double bonds [12]. Microalgae biomass for biodiesel synthesis has been produced using either pure or mixed cultures in photoautotrophic, heterotrophic or mixotrophic growth systems. Phototrophic cultivation may be performed in open or closed systems, while heterotrophic growth is maintained in fermenters [25]. The most important factor for efficient production of lipids is the carbon source: for example, *Chlorella prothothecoides* can grow photoautotrophically or heterotrophically, however usage of acetate or glucose as carbon source may yield much higher biomass and lipid content. By utilizing the less expensive carbon sources such as ethanol, glycerol or fructose, may be considered to reduce the production cost [55]. Table 6 shows the oil content in microalgae.

Table 7
Application of lipase enzyme on algae for production of biodiesel.

Raw material/oil	Name of enzyme	Lipase origin	Immobilized method	Carrier	Solvent used	Yield/conversion	Oil extraction method	References
Microalgae (*Chlorella prototothecoides*)	Lipase	*Candida sp.* 99–125	Adsorption	Macroporous resin	Methanol	98%	–	[62]
Microalgae (*Chlorella vulgaris ESP-31*)	Lipase	*Burkholderia sp.C20*	Covalent binding	Alkyl-grafted Fe_3O_4- SiO_2	Methanol	97.3 wt% oil	Sonication	[44]
Microalgae (*Chlorella sp. KR-1*)	Novozyme 435	*Candida antartica*	Adsorption	Macroporous resin	DMC mixture	> 90%	DMC and methanol mixture (7:3 (v/v))	[63]
Microalgae (*Chlorella pyrenoidosa*[b], *Chlorella vulgaris*, *Botryococcus braunii* (BB763, BB764))	Novozyme 435[a]	*Candida antartica*[a], *Penicillium expansum*	Adsorption[a]	Acrylic resins[a]	Ionic liquid [BMIm][PF$_6$], *tert*-butanol	90.7% & 86.2% in ionic liquid; 48.6% & 44.4% in *tert*-butanol	Soxhlet[b] (solvent: hexane)	[26]
Microalgae (*Chlorella prototothecoides*)	Lipase	*Candidia sp.* 99–125	Adsorption	–	Methanol	98.15%	Soxhlet (solvent: *n*-hexane)	[45]

[a] Novozyme 435 from *Candida antarctica* lipase was immobilized on acrylic resins.
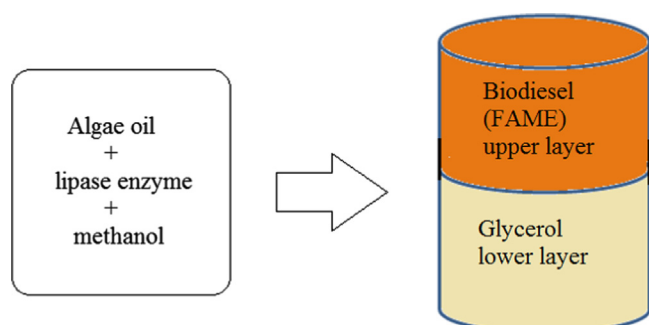[b] Algae from *Chlorella pyrenoidosa* extracted by Soxhlet using hexane as solvent.

Fig. 4. Illustration of lipase esterification of algae oil into biodiesel and by product (glycerol).

Various extraction methods and transesterifications of algae oil into FAME (biodiesel) are reported in the literature study [30,61]. There are limited study for enzyme transesterification from algae compared to other feedstocks such as jatropha oil, soybean oil and palm oil as reported in Table 4. However, studies show that the enzyme from lipase is excellent for various vegetable oil conversions to methyl ester. There are more than 90% yields of crude oil could be obtained with conversion conditions of 35–55 °C at atmospheric pressure where the molar ratio of oil to alcohol is 3:1 to 10:1 [35]. Table 7 shows the application of lipase enzyme on algae for biodiesel production. *Chlorella* sp. are the most popular and versatile algae used in biodiesel production because of its higher production yield. For example, Xiong et al. [62] used lipase from *Candida* sp. 99–125 to catalyze oil from *Chlorella prototothe-coides* to produce biodiesel, and the conversion rate reached 98% after 12 h.

Fig. 4 illustrates the lipase transesterification of algae oil into biodiesel with methanol as the acyl acceptor. Two layers of solution will be attained after centrifugation, whereby the upper layer is the FAME produced while the by product, glycerol remained in the lower layer. Another study by Tran et al. [44] reported that biodiesel conversion of 97.3 wt% oil obtained from esterification of microalgae oil (*Chlorella vulgaris* ESP-31) using immobilized *Burkholderia* sp. C20 lipase as shown in Table 7. This lipase was immobilized on a hybrid nanomaterial ($Fe_3O_4-SiO_2$) with a long chain alkyl group used as supporters. As a result, biodiesel could be produced from wet algae biomass without significant dewatering and oil extraction with less cost. The use of ionic liquid for example 1-butyl-3-methylimidazolium hexa-fluorophosphate, [BMIm][PF$_6$] also demonstrates an efficient

conversion of biodiesel from microalgae *Chlorella pyrenoidosa*, *Chlorella vulgaris*, and two strains from *Botryococcus braunii* (BB763, BB764) [26]. Two sources of lipase enzyme were utilized in this study which are from *C. antarctica* (Novozyme 435) and *Penicillium expansum*. The higher conversion of biodiesel was obtained for microalgae oil in ionic liquid, 90.7% and 86.2% for *P. expansum* and Novozyme 435 lipase, respectively. However, lower conversion yield was attained which are 48.6% and 44.4% in *tert*-butanol for *P. expansum* and *C. antarctica* (Novozyme 435) lipase, respectively.

As discussed in Section 2.1, high concentration of methanol will decrease the activity and affect stability of lipase. Moreover, phospholipid content in oil feedstock also has been found to affect the lipase activity [64]. Phospholipids content of microbial oils are much higher if compared with vegetable oils. For instance, lipids extracted from fungi or algae are reported to comprise more than 10% phospholipids. Presence of 1% phospholipids could cause the irreversible deactivation of immobilized lipase in some reactions. Meanwhile, 0.5% phospholipids content in oil will reduce reaction rate and yield of biodiesel during immobilized lipase-mediated biodiesel production [65]. In brief, the higher the phospholipid content in the oil, the lower is the methyl ester yield [66].

Methanol and *n*-hexane had been reported as the acyl acceptor in enzymatic transesterification. However, *n*-hexane tends to reduce the reaction rate due to dilution of reactants and addition of mass transfer resistances [47]. On the other hand, dimethyl carbonate (DMC) solvent has been implemented in conversion of microalgae oil from *Chlorella* sp. KR-1 into FAME by using Novozyme 435 as the catalyst [63]. DMC can be a good choice of solvent for extracting oil from microalgae biomass because DMC has a good solvent extraction capacity and it remains stable when heated and condensed, without decomposition. Herein, DMC was used as the reaction medium and acyl acceptor. As a result, more than 90% conversion was obtained with 293.82 mg FAMEs/g biomass in 6 h of reaction time at 60 °C in the presence of 0.2% (v/v) water.

3. Application of enzyme for bioethanol production from algae

Bioethanol is another substitute biofuel that could be produced from biological resources. Furthermore, bioethanol is less toxic, readily biodegradable and its use produces fewer air-borne pollu-tants than petroleum fuel [67]. Bioethanol also can be employed

Table 8
Comparison major terrestrial bioethanol crops and macroalgae [73].

Parameter	Wheat (grain)	Corn (kernel)	Sugar beet	Sugarcane	Macroalgae
Average world yield (kg ha^{-1} year^{-1})	2800	4815	47,070	68,260	730,000
DW of hydrosable carbohydrates (kg ha^{-1} year^{-1})	1560	3100	8825	11,600	40,150
Potential volume of bioethanol (kg ha^{-1} year^{-1})	1010	2010	5150	6756	23,400

to replace octane enhancers such as methylcyclopentadienyl manganese tricarbonyl (MMT) and aromatic hydrocarbons such as benzene or oxygenates such as methyl tertiary butyl ether (MTBE) [68].

Currently, bioethanol is mainly descended from sucrose and starch crops (e.g., sugarcane and corn) as well as lignocellulosic materials (e.g., rice straw and switchgrass) [69]. First generation of bioethanol appear to be the most feasible short-term alternative to fossil fuels due to efficient production technology. Sugarcane is the main feedstock for bioethanol production in Brazil, whereas corn and sugar beet are the major resources in United States and European Union, respectively [67]. Yet, the increase production of bioethanol from edible crops such as sugarcane and corn, leave an impact on the food prices, increasing deforestation, higher usage of arable land and high water usage [70]. Therefore, second generation of bioethanol has emerged in which it is derived from waste biomass (lignocellulose feedstocks). The major drawbacks of the latter are high lignin content in the lignocellulosic biomass, making the saccharification process very difficult and high cost of converting lignocellulosic materials into ethanol [69]. Moreover, the use of lignocellulosic material always resulted in low yield with high cost of the cellulase enzyme for the hydrolysis process. The high cost of cellulase enzyme is a major factor in the enzymatic saccharification of agricultural biomass, which contains lignin. Thus, even though starchy or cellulosic materials are cheaper than sugar-containing raw materials, the upstream process is considered as the main constraint.

The weaknesses of the first and second generation of bioethanol make microalgae as one of the important feedstock for bioethanol. Bioethanol produced from microalgae feedstock could be an alternative, as algae biomass is less resistant to conversion into simple sugars than plant biomass [70]. The bioethanol production from microalgae is primarily obtained through fermentation of starch, sugar and cellulose contained in microalgae biomass. Several species of microalgae have the ability to produce high levels of carbohydrates instead of lipids as reserve polymers. These species are the most suitable sources for production of bioethanol to be converted into sugars. It has been estimated that approximately 5000–15,000 gal of ethanol/acre (46,760–140,290 L/ha) could be produced from microalgae annually which is larger than other feedstocks [71].

The production of bioethanol involves a wide process and differs depending on the type of biomass. The production process includes downstream and upstream process, namely pre-treatment, saccharification, fermentation and product recovery. Pre-treatment is a vital process for the fermentable sugars to be released and made available for the fermentation process [72]. Biomass can be pre-treated in three different mechanisms; physical, biological and chemical. Basically, the microalgae will be lysed by mechanical equipment or an enzyme. Then, yeast of *Saccharomyces cerevisiae* (for example) is added to begin the fermentation. The fermentation product is called bioethanol [71].

Microalgae and macroalgae have been considered as the third generation of biodiesel and bioethanol because of their pleasant characteristics. Some of the microalgae species have high carbohydrate content, in terms of starch and cellulose, which is the required substrates for bioethanol production [69]. It was reported

Table 9
The list of carbohydrate content of algae.

Algae	Carbohydrate content (%)	References
Ulva fasciata	38.5–47.5	[75]
Chlorococcum humicola	32.52	[72,76]
Sargassum sp.	41.81	[77]
Sargassum kushimonte	42.89	[73]
Sargassum cristaefolium	46.78	[73]
Gracilaria verrucosa	41–43	[78]
Dunaliella tertiolecta	37.8	[79]
Chlorella vulgaris	37–55	[67,71,74]
Ulva lactuca,	54.3	[80]
Gelidium amansii	77.2	[80]
Laminaria japonica	51.9	[80]
Sargassum fulvellum	39.6	[80]

that the carbohydrate content of microalgae is around 70–72% where starch governed the carbohydrate content and could be up to 60% by dry weight depending on the culture condition [35]. Microalgae have been applied for biodiesel production, the algae biomass left after oil extraction can be fermented into ethanol or methane or integrated in livestock feed or applied as organic fertilizer due to high ratio of N:P [55].

In general, algae are classified into two categories; microalgae and macroalgae; based on their morphology and size. Microalgae are microscopic photosynthetic organisms, many of which are unicellular compared to macroalgae, for example kelps, are composed of multiple cells which organize to structures resembling roots, stems, and leaves of higher plants [67]. Table 8 compares macroalgae with conventional terrestrial bioethanol feedstocks, such as sugarcane, corn, and wheat. A lot of benefits are associated with microalgae as the source of bioethanol such as: (i) rich with carbohydrate, (ii) less resistant to conversion into simple sugars, (iii) higher growth rate, (iv) short time span of algae cells harvest, (v) higher fixation rate of CO_2 than terrestrial plants, (vi) can utilize CO_2 emitted from petroleum-based power stations or other industrial sources, (vii) reduce emission of greenhouse gas, (viii) less toxic, (ix) do not require soil, (x) can be grown all year around in some climates, (xi) can be grown in the absence of fresh water and perform very well in waste or saline water streams and (xii) lower energy requirement [69,70,73].

Algae has low percentage of lignin and hemicellulose compared to other plants and it has a significant potential for bioethanol [71]. Microalgae like *Chlorella, Chlamydomonas, Dunaliella, Scenedesmus,* and *Tetra-selmis* have been shown to accumulate a large amount of carbohydrates ($>50\%$ of the dry weight) of starch and glycogen [67]. Table 9 listed some of the carbohydrate content of micro/macroalgae. *Chlorella* sp. which have been reported as the common sources of bioethanol due to high content of carbohydrate. *Chlorella vulgaris* is among the most popular microalgae reported with high carbohydrate content, 37–55% of its dry weight [71,74]. In addition, another species of *Chlorococum* also utilized as a substrate for bioethanol production under different fermentation conditions. As a result, a maximum bioethanol concentration of 3.83 g/L was obtained from 10 g/L of lipid-extracted microalgae debris [71].

The hydrolysis of microalgae could be catalyzed by several methods, for instance physical (milling, grinding, and pyrolysis), chemical (acid/alkali) or biological (enzyme). Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific and the products are usually reducing sugars including glucose [77]. Different enzymes were used in the hydrolysis step and the process is influenced by numerous factors including cellulose crystallinity, substrate surface area, cell wall thickness, porosity, mass transfer, and hemicellulose or lignin contents [76]. While microalgae have been reported to have no lignin composition, it can be categorized as a cellulosic based material and the cellulase enzyme could be applied to hydrolyze microalgae biomass. However, the utilization of microbes or enzymes to degrade the biomass has been associated with low hydrolysis rate which prolongs the completion time of the process [72].

The chemical method has been successfully proven for various biomass, whereas the physical method consume high energy and is not preferred for commercial use. In addition, acid pre-treatment process (hydrochloric acid and sulfuric acid) is more efficient in converting cellulosic materials contrasted to the alkaline (lime and sodium hydroxide) [72]. Acid pre-treated system gave almost twice yield, 58 wt% of yield (g ethanol/g microalgae) whereas the $SCCO_2$ pre-treated system yielded around 38 wt% (g ethanol/g microalgae) [72]. Diluted acid hydrolysis is probably the most commonly applied method among the chemical hydrolysis method. Table 10 describes the advantages of enzymatic hydrolysis compared to diluted-acid hydrolysis. Enzymatic hydrolysis work in mild condition (e.g. pH 4.5–5.0 and temperature 40–50 °C) compared to diluted-acid hydrolysis which operated under high temperature and low pH. Enzymatic hydrolysis is also capable to hydrolyze almost 100% of cellulose [81].

Several applications of cellulase enzyme on micro- and macro-algae are summarized in Table 11. Macroalgae from *Ulva fasciata* with carbohydrate content of $43 \pm 4.5\%$ (on dry weight) has been successfully producing bioethanol by enzymatic reaction with 88.2% fermentation efficiency. Four commercial cellulase enzymes were used to catalyze the reaction. The highest bioethanol content was obtained from Cellulase 22119 with 0.45 (g/g sugar) [75]. Another enzyme, *β-glucosidase* or also known as Novozyme 188, commonly used simultaneously with cellulase enzyme as a supplement enzyme. *β-glucosidase* functioned to hydrolyze the cellobiose which is an inhibitor of cellulase activity [77]. Macroalgae from *Sargassum* spp and *Gracilaria verrucosa* has been utilized in production of bioethanol by commercial cellulase and *β-glucosidase.* As a result, 89% conversion from *Sargassum* spp

Table 10
Comparison between dilute-acid and enzymatic hydrolyzes [81].

Comparing variable	Dilute-acid hydrolysis	Enzymatic hydrolysis
Mild hydrolysis conditions	No	Yes
High yields	No	Yes
Product inhibition during hydrolysis	No	Yes
Formation of inhibitory by-products	Yes	No
Cost of catalyst	Low	High

Table 11
The summary of bioethanol from algae by enzymatic hydrolysis

	Hydrolysis				Fermentation			
Raw material/oil	Name of enzyme	Origin	Solvent used	Most Efficient	Cells used	Medium	Ethanol yield/conversion	References
Macroalgae Green seaweed *Ulva fasciata* Delile *(Chlorophyceae)*	Cellulase (Viscozyme L), Cellulase 22128, Cellulase 22119, and Cellulase 22086	–	Sodium acetate buffer, ph 4.8	Cellulase 22119	Yeast strain *Saccharomyces cerevisiae* (MTCC No. 180)	YEPD medium	0.45 g/g sugar	[75]
Macroalgae Brown seaweed *Sargassum sp.*	Cellulase[a] and β-glucosidase [b] (cellobiase, Novozyme 188)	*Trichoderma reeseii* ATCC 26921[a], *Aspergillus niger* [b]	0.1M Citrate buffer, pH 4.8	Both	Yeast strain *Saccharomyces cerevisiae*	0.9 g/Li (NH4) 2SO4 and 0.375 g/Li yeast extract	89%	[77]
Red algae *Gracilaria verrucosa*	Cellulase [c] and β-glucosid ase [d] (Novozyme 188)	*Trichoderma reesei* [c] ATCC 26921, *Aspergillus niger* [d]	0.05 M Citrate phosphate buffer, pH 5.0	Both	Yeast strain *Saccharom yces cerevisiae* HAU	Enzymatic hydrolysate, 3g/L yeast extract and 0.25 g/L $(NH_4)_2HPO_4$	0.43 g/g sugars	[78]
Microalgae *Chlorococum humicola*	Cellulase	*Trichod erma. reesei* ATCC 26921	Sodium acetate buffer, pH 2.5-7.5	-	-	-	64.2% (w/w) of glucose	[76]
Microalgae *Dunaliella tertiolecta* LB999	Cellulase (Celluclast 1.5L, Novoprime B957), Amyloglucosidase (AMG 300L), Viscozyme L	–	0.1 M Sodium acetate buffer, pH 5.5	AMG 300L	Yeast strain *Saccharomyces cerevisiae* YPH500 (ATCC 76626)	Yeast extract, 3 g/L; malt extract, 3 g/L; peptone 5 g/L; dextrose 10 g/L	0.14 g ethanol/g residual biomass and 0.44 g ethanol/g glucose	[79]
Green algae *Ulva lactuca,*	Celluclast1.5L, Viscozyme L, Novoprime 959, Novoprime 969, Amyloglucosidase (AMG 300L)	–	0.05 M Citrate buffer, pH 5.5	Combination of Celluclast1.5L and Viscozyme L	Yeast strain *Saccharomyces cerevisiae* (ATCC #24858), recombinant *E. coli*	MGYP medium	0.4 g ethanol per g of carbohydrate when cultured in *L. japonica* hydrolysate	[80]

Table 11 (*continued*)

	Hydrolysis				Fermentation			
Raw material/oil	Name of enzyme	Origin	Solvent used	Most Efficient	Cells used	Medium	Ethanol yield/ conversion	References
Red algae *Gelidium amansii* Brown algae *Laminaria japonica, Sargassum fulvellum*					KO11 (ATCC #55124), a derivative of *E. coli* B (ATCC #11303)			

[a,b] The samples were incubated at 50 ± 1 °C, 100 rpm for a period of 48 h. The samples were hydrolysed using 50 FPU cellulase/g biomass and 250 CBU cellobiase/g biomass.

[c,d] The samples were incubated at 50 °C, 150 rpm for a period of 2 h. Then, the suspension was supplemented with cellulase (20 FPU/g dry substrate)and β-glucosidase (60 U/g dry substrate).

[77] and 0.43 g/g sugar from *G. verrucosa* [78] were being reported. The optimal saccharification efficiency ($87.58 \pm 1.71\%$) was obtained after 36 h with saccharification rate of 1.08 ± 0.02 g/L/h and was significantly higher than the saccharification efficiency of other seaweeds such as *U. pertusa*, *Alaria crassifolia* and *Gelidium elegans* which may be because of the availability of fermentable carbohydrate in the *Gracilaria* pulp, optimum saccharification conditions and the enzyme efficiency [78].

Meanwhile, Harun et al. [76] investigated the effect of various temperature (28 °C, 40 °C, 60 °C), pH (2.5–7.5) and biomass concentration (0.1, 0.08, 0.04, 0.02; [E]/[S]) towards microalgae *Chlorococum humicola*. Consequently, the highest glucose yield of 68.2% (w/w) was obtained when the hydrolysis process was performed using 10 g/L of biomass at pH 4.5 and 40 °C. It could be concluded that each enzyme has its own characteristics and behave well in their optimum condition from this study. Furthermore, chemo-enzymatic saccharification and bioethanol fermentation of the residual biomass of *Dunaliella tertiolecta* after lipid extraction for biodiesel production were investigated using AMG 300L [79]. From the investigation, 21.0 mg/mL of reducing sugar with a yield of 42.0% (w/w) based on the residual biomass at pH 5.5 and 55 °C was produced. Besides, bioethanol was produced from the enzymatic saccharification products without additional pretreatment by *S. cerevisiae* with yields of 0.14 g ethanol/g residual biomass and 0.44 g ethanol/g glucose produced from the residual biomass.

Most of the researches reported previously, have utilized yeast from *S. cerevisiae* in their fermentation reaction. Kim et al. [80] demonstrated the possibility of ethanogenic *Escherichia coli* KO11 to catalyze the fermentation of algae. Four species of algae consisting of *Ulva lactuca*, *Gelidium amansii*, *Laminaria japonica*, and *Sargassum fulvellum* were used to study the effect of *E. coli* in production of bioethanol. Several enzymes were utilized to hydrolyze the algae cells and the highest sugar release of up to 56.6% was observed when Viscozyme L and Celluclast 1.5L (1:1 by volume) were added together, suggesting a synergistic effect [80]. Furthermore, the capability of *E. coli* KO11 was proven to utilize mannitol which is present in high amounts in the hydrolysate since the yeast was unable to ferment mannitol. Hence, 0.4 g ethanol/g of sugars produced successfully after fermentation.

4. Conclusion

Algae could be found abundantly and it is recorded as the earlier species on earth. Application of algae for production of biofuel is not new, yet it needs improvements from some aspects such as the materials used, processes, and separation of product. Based on this review, it seems that enzymatic transesterification could be improved to achieve higher yield of biodiesel with less contamination, low cost and simple operation. A lot of areas could be expanded in terms of solvent used, enzyme immobilization method, extraction method and separation of the products. Immobilization of enzyme is an important parameter that influences the efficiency of lipase used to maintain stability. Enzymatic engineering may help increase the product yield by creating recombinant enzyme that efficiently breaks triglycerides into FAME and glycerol with certain properties such as temperature resist, stable, compatible with solvent used and higher yield.

The high potential of micro- and macro-algae as the main producer for bioethanol makes algae one of the important feedstock besides food crops and lignocellulosic materials. All trapped starch or carbohydrates inside the algae cells should be taken out for further fermentation process. Improvement of cellulase enzyme in low cost and efficiency will enhance the bioethanol production further. Besides that, the development of efficient and cost-effective fermentation processes, and post fermentation markets for macroalgae waste biomass requires further research. The main concern in production of biofuel by enzymatic reaction is the efficiency of the enzyme. The study of enzyme efficiency by manipulating the immobilization method for biodiesel production need to be carried out. Immobilization method is the key for high conversion of biodiesel and it requires the selected raw material to contain a high oil content to have higher product yield.

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